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# A potential mechanism for selective control of cap-independent translation by a viral RNA sequence in cis and in trans

## Abstract

Highly efficient cap-independent translation initiation at the 5'-proximal AUG is facilitated by the 3' translation enhancer sequence (3'TE) located near the 3' end of barley yellow dwarf virus (BYDV) genomic RNA. The role of the 3'TE in regulating viral translation was examined. The 3'TE is required for translation and thus replication of the genomic RNA that lacks a 5' cap (Allen et al., 1999, *Virology* 253:139-144). Here we show that the 3'TE also mediates translation of uncapped viral subgenomic mRNAs (sgRNA1 and sgRNA2). A 109-nt viral sequence is sufficient for 3'TE activity in vitro, but additional viral sequence is necessary for cap-independent translation in vivo. The 5' extremity of the sequence required in the 3' untranslated region (UTR) for cap-independent translation in vivo coincides with the 5' end of sgRNA2. Thus, sgRNA2 has the 3'TE in its 5' UTR. Competition studies using physiological ratios of viral RNAs showed that, in trans, the 109-nt 3'TE alone, or in the context of 869-nt sgRNA2, inhibited translation of genomic RNA much more than it inhibited translation of sgRNA1. The divergent 5' UTRs of genomic RNA and sgRNA1 contribute to this differential susceptibility to inhibition. We propose that sgRNA2 serves as a novel regulatory RNA to carry out the switch from early to late gene expression. Thus, this new mechanism for temporal control of translation control involves a sequence that stimulates translation in cis and acts in trans to selectively inhibit translation of viral mRNA.

## Keywords

3' untranslated region, barley yellow dwarf virus, subgenomic RNA, translational switch, Genetics and MCDB Programs

## Disciplines

Agricultural Science | Genetics | Plant Pathology

## Comments

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# A potential mechanism for selective control of cap-independent translation by a viral RNA sequence in *cis* and in *trans*

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## ABSTRACT

Highly efficient cap-independent translation initiation at the 5'-proximal AUG is facilitated by the 3' translation enhancer sequence (3'TE) located near the 3' end of barley yellow dwarf virus (BYDV) genomic RNA. The role of the 3'TE in regulating viral translation was examined. The 3'TE is required for translation and thus replication of the genomic RNA that lacks a 5' cap (Allen et al., 1999, *Virology* 253:139–144). Here we show that the 3'TE also mediates translation of uncapped viral subgenomic mRNAs (sgRNA1 and sgRNA2). A 109-nt viral sequence is sufficient for 3'TE activity *in vitro*, but additional viral sequence is necessary for cap-independent translation *in vivo*. The 5' extremity of the sequence required in the 3' untranslated region (UTR) for cap-independent translation *in vivo* coincides with the 5' end of sgRNA2. Thus, sgRNA2 has the 3'TE in its 5' UTR. Competition studies using physiological ratios of viral RNAs showed that, *in trans*, the 109-nt 3'TE alone, or in the context of 869-nt sgRNA2, inhibited translation of genomic RNA much more than it inhibited translation of sgRNA1. The divergent 5' UTRs of genomic RNA and sgRNA1 contribute to this differential susceptibility to inhibition. We propose that sgRNA2 serves as a novel regulatory RNA to carry out the switch from early to late gene expression. Thus, this new mechanism for temporal control of translation control involves a sequence that stimulates translation *in cis* and acts *in trans* to selectively inhibit translation of viral mRNA.

**Keywords:** 3' untranslated region; barley yellow dwarf virus; subgenomic RNA; translational switch

## INTRODUCTION

RNA viruses use a variety of strategies to compete aggressively with host mRNAs for translational machinery and to regulate gene expression in ways that favor maximum viral accumulation. One such strategy is cap-independent translation (Jackson & Kaminski, 1995; Sarnow, 1995). This allows the virus to avoid cellular cap-mediated translational control mechanisms, sometimes shutting down cap-dependent translation, and it obviates the need to encode a capping enzyme or acquire a cap via cellular enzymes. The 5' cap structure, m<sup>7</sup>G(5')ppp(5')N, is required for efficient initiation and regulation of translation of cellular mRNAs (Sonnenberg, 1996; Sachs et al., 1997). Via initiation factors, the 5' cap recruits the 40S ribosomal subunit that scans in the 3' direction, initiating protein synthesis at the first (and occasionally second) AUG codon (Kozak, 1989).

Many viral RNAs lack a 5' cap. For example, the genomes of picornaviruses (Pelletier & Sonenberg, 1988; Jackson & Kaminski, 1995) and pestiviruses (Wang et al., 1993) have a highly structured 5' untranslated region (250–600 nt) that acts as an internal ribosome entry site (IRES) that facilitates cap-independent translation. IRESs have also been found in other viruses and a few exceptional cellular mRNAs (Sarnow, 1995). All these *cis*-acting, internal initiation signals are located in the 5' untranslated region (UTR), so that after the ribosome binds it scans in the 3' direction until the start codon is reached, in accordance with the scanning model. Although cap-independent translation mechanisms have been scrutinized, little is known about how RNA viruses regulate their own cap-independent translation over time. Here we provide evidence that suggests a novel mechanism by which the RNAs of BYDV may interact to regulate cap-independent expression of viral genes over the course of an infection cycle.

The 3' end of mRNA also participates in translation initiation (Gallie, 1991; Tarun & Sachs, 1995; Jacobson, 1996; Sachs et al., 1997). The poly(A) tail interacts synergistically with the 5' cap in stimulating translation *in vivo* (Gallie, 1991; Tarun et al., 1997; Preiss

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& Hentze, 1998). In viral RNAs that lack a 3' poly(A) tail, other sequences in the 3' UTR may stimulate translation (Leathers et al., 1993). The RNAs of barley yellow dwarf virus (BYDV; Allen et al., 1999) and satellite tobacco necrosis virus (STNV; Lesnaw & Reichmann, 1970) lack both a 5' cap and a poly(A) tail. The RNAs of these viruses each contain a different sequence in the 3' UTR that confers efficient cap-independent translation on uncapped mRNA (Danthinne et al., 1993; Timmer et al., 1993; Wang & Miller, 1995; Wang et al., 1997; Meulewaeter et al., 1998).

BYDV is in the genus *Luteovirus* of the family *Luteoviridae*. Members of the family *Luteoviridae* have a single stranded, positive-sense RNA genome of 5.6 to 5.7 kb encoding about six open reading frames (ORFs) (Mayo & Ziegler-Graff, 1996; Miller, 1999). Viruses in the genus *Polyovirus* of the family *Luteoviridae* have a VPg linked to the 5' terminus of the genome (Mayo et al., 1982; Murphy et al., 1989), whereas BYDV RNA has neither a VPg (Shams-bakhsh & Symons, 1997) nor a 5' cap (Allen et al., 1999). During its life cycle, BYDV produces three subgenomic RNAs (sgRNAs) that are 3' coterminal with genomic RNA (gRNA) (Fig. 1) (Kelly et al., 1994; Mohan et al., 1995; Miller et al., 1997). The ORFs (1 and 2) in the 5' half of genome are translated from gRNA (Wang & Miller, 1995). ORF 2, which encodes the RNA-dependent RNA polymerase, is translated by ribosomal frameshifting from ORF 1 to generate a 99-kDa fusion product (Di et al., 1993). ORFs 3, 4, and 5 code for the coat protein, movement protein, and an aphid transmission function, respectively (reviewed by Miller, 1999). All three ORFs are translated only from sgRNA1 (Fig. 1) (Brown et al., 1996). ORF 4 is translated by leaky scanning (Dinesh-Kumar & Miller, 1993) and ORF 5 by in-frame readthrough of the ORF 3 stop codon (Brown et al., 1996). Subgenomic RNA2 (sgRNA2) may serve as a message for ORF 6 (Kelly et al., 1994) and as a *trans*-regulator of viral translation (this report).

Previously, we reported that a 3' translation enhancer (3'TE) sequence, located 5 kb downstream from the 5' terminus of BYDV genomic RNA (Fig. 1) confers

efficient translation initiation at the 5'-proximal AUG of uncapped RNA (Wang & Miller, 1995; Wang et al., 1997). A 109-nt 3'TE sequence is sufficient in wheat germ extracts, but a longer portion of the viral genome is needed for full cap-independent translation *in vivo* (Wang et al., 1997). A functional 3'TE is necessary for BYDV RNA replication because it is required for translation of the replicase (Allen et al., 1999). Left unanswered has been the role of the 3'TE in subgenomic RNA translation and in the virus life cycle in general. Here we provide evidence that the 3'TE can function both to facilitate translation of viral genes *in cis*, and to specifically inhibit translation *in trans*. Thus, it may act as a novel *trans*-regulator of viral gene expression.

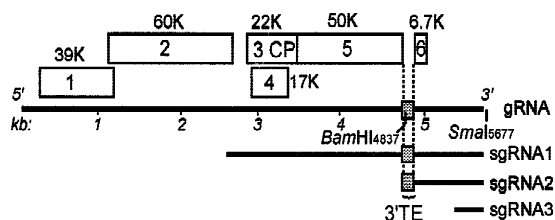
## RESULTS

### The 5' extremity of the 3' BYDV sequence needed for cap-independent translation *in vivo* coincides with the 5' end of sgRNA2

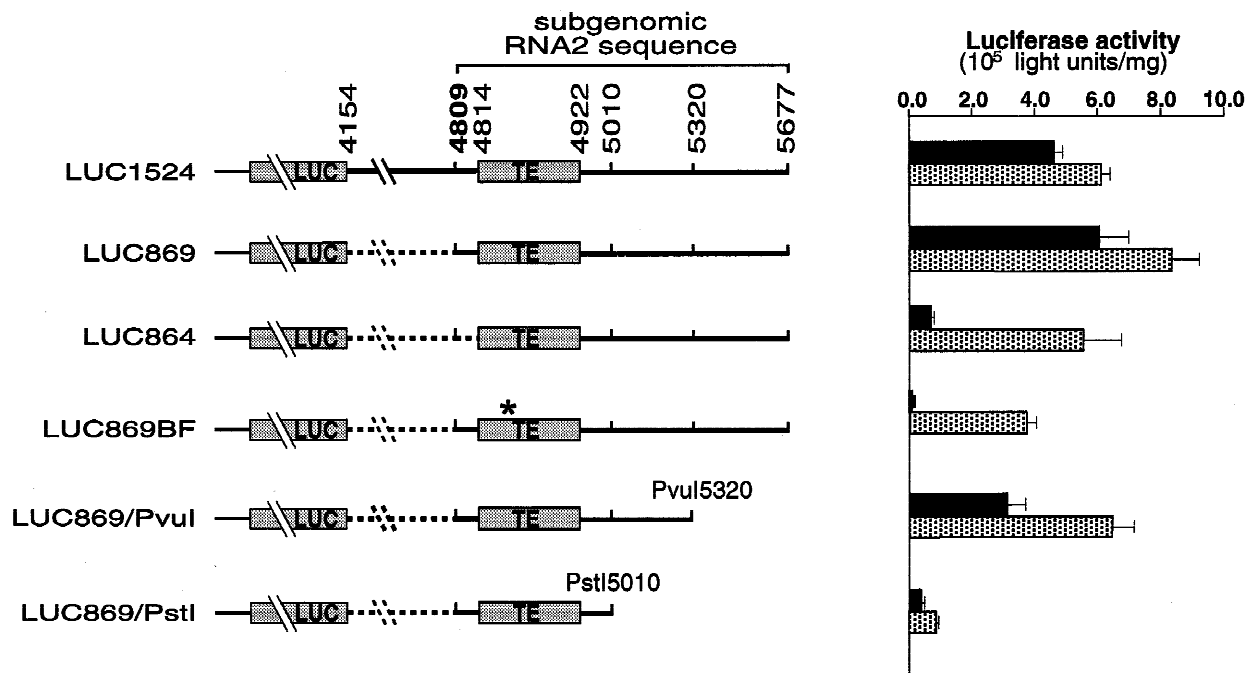
Previously, we showed that the 109-nt 3'TE (bases 4814–4922) defined in wheat germ extract was not sufficient to give full cap-independent translation *in vivo*. The 3'-terminal 1,162 nt of the BYDV genome, which encompasses the 109-nt 3'TE, gave very efficient cap-independent translation of a  $\beta$ -glucuronidase (GUS) reporter gene in oat protoplasts (Wang et al., 1997). To more precisely map the sequence(s) needed for full activity *in vivo*, constructs were made containing smaller portions of the viral genome in the 3' UTR of a reporter gene, this time using the firefly luciferase reporter gene (Fig. 2).

mRNAs containing sequence from the 3' end of the BYDV genome spanning bases 4154–5677 (LUC1524) or 4809–5677 (LUC869) in the 3' UTR translated efficiently in the presence or absence of a 5' cap. However, uncapped mRNA containing nt 4814–5677 in the 3'UTR (LUC864) had sharply reduced translation, compared to its capped counterpart and uncapped LUC869 (Fig. 2). Base 4809 (5' end of BYDV sequence in LUC869) corresponds precisely to the 5'-terminal base of sgRNA2 (Kelly et al., 1994), whereas base 4814 (5' end of BYDV sequence in LUC864) corresponds to the 5' end of the wheat germ-defined 3'TE. As a negative control, a mutant version of LUC869 (LUC869BF) that contains a four-base duplication made by filling the *Bam*HI<sub>4837</sub> site gave extremely low luciferase activity. This mutation was shown previously to obliterate cap-independent translation *in vitro* and *in vivo* (Wang et al., 1997).

Efficient *in vivo* translation of capped forms of all constructs with the UTR extending to the 3' end of the viral genome (nt 5677, Fig. 2) indicates that the poor expression of uncapped LUC864 RNA and LUC869BF RNAs was due to loss of the cap-independent transla-



**FIGURE 1.** Genome organization of BYDV. Open reading frames are indicated both by numbers and by molecular weight in kilodaltons (K). Scale of RNA is indicated in kilobases (kb). Positions of selected restriction enzyme sites are indicated. Bold lines indicated genomic (gRNA) and subgenomic (sgRNA) RNAs. Shaded box indicates 109-nt 3'TE defined *in vitro*.



**FIGURE 2.** Deletion mapping of the BYDV 3' UTR sequences involved in cap-independent translation in vivo. Uncapped transcripts encoding luciferase (LUC) flanked by the BYDV 5' UTR and indicated portions of the 3' end of the BYDV genome were electroporated into oat protoplasts. Luciferase activity from uncapped transcripts (black bars) and capped transcripts (stippled bars) for each construct is indicated. All RNAs were from *Sma*I-linearized plasmids (nt 5677), except LUC869/PstI and LUC869/PvuI, which were from pLUC869 linearized with the indicated restriction enzymes. Asterisk indicates location of *Bam*HI<sub>4837</sub> fill-in mutation in LUC869BF. Assays were performed in triplicate with standard error bars shown. Numbers on map of 3' UTR are the positions in the BYDV genome.

tion function and not some other process unrelated to translation initiation. Deletion of the 357 nt at the 3' end of the viral sequence by truncation at the *Pvu*I site only slightly reduced translation of uncapped mRNA (LUC869/*Pvu*I, Fig. 2). In contrast, deletion of an additional 310 nt (*Pst*I truncation) reduced translation of both capped and uncapped mRNAs by an additional sevenfold (LUC869/*Pst*I, Fig. 2). Thus, a sequence between nt 5010 and 5320 is required in the 3'UTR for translation and/or mRNA stability, but it is not specific for cap-independent translation.

### The 3'TE is required for efficient translation of uncapped subgenomic RNAs

Why is the 3'TE located at the 3' end of the viral genome, instead of the more "conventional" 5' end? One possibility is that this allows one genomic copy of the 3'TE sequence to facilitate cap-independent translation of genomic and subgenomic RNAs without needless duplication at the 5' UTR of each RNA. The 3'TE is located in the 3' UTR of sgRNA1 and in the 5' UTR of sgRNA2 (Fig. 1), so it may facilitate cap-independent translation of both RNAs. To investigate this, full-length sgRNA1 (bases 2670–5677; Kelly et al., 1994) was translated. When intact 3'TE was present, translation of uncapped sgRNA1 transcripts was almost as

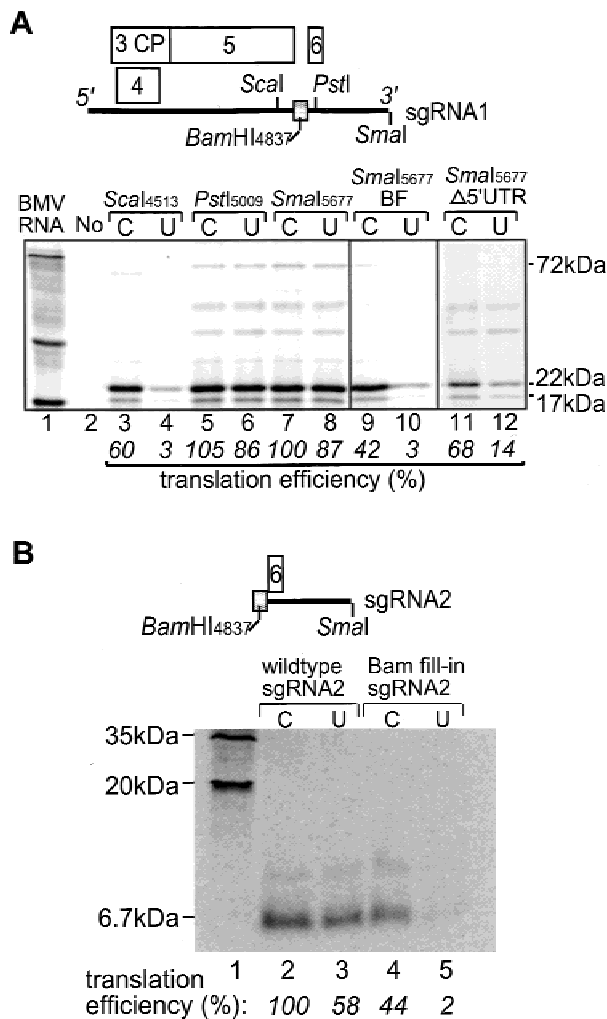
efficient as translation of their capped counterparts (Fig. 3A, lanes 5–8). Transcripts lacking the 3'TE or containing the *Bam*HI<sub>4837</sub> fill-in mutation gave about 30-fold less translation product than the 3'TE-containing transcripts (Fig. 3A, lanes 3–4, 9–10), and 14 to 18-fold less than the capped form of the same mRNA. Thus, the 3'TE functions similarly on both the genomic RNA and sgRNA1.

Previously, we showed that replacement of the genomic RNA 5' UTR with either of two different non-BYDV sequences knocked out cap-independent translation (Wang & Miller, 1995; Wang et al., 1997), yet the 5' UTR of sgRNA1 shows little sequence similarity to that of genomic RNA. Thus, we investigated the role of the 5' UTR of sgRNA1 in 3'TE-mediated cap-independent translation. Deletion of 99 bases from the 5' end of the 188-nt 5' UTR of sgRNA1 decreased the translation of uncapped mRNA by fivefold, even in the presence of wild-type 3'TE (Fig. 3A, lanes 11–12). Capping of this 5' truncated version of subgenomic RNA restored most of its translation efficiency. Therefore, sequence(s) within the 5'-terminal 99 bases of the sgRNA1 5' UTR is necessary for full function of the 3'TE.

The 3'TE conferred cap-independent translation on sgRNA2 in which it is located in the 5' UTR. Translation of ORF 6 from sgRNA2 was determined by comparing



## Control of cap-independent translation by RNA



**FIGURE 3.** Cap-independent translation of capped (C) and uncapped (U) subgenomic RNAs. **A:** Wheat germ translation products of sgRNA1 (map at top), which was transcribed from pSG1 linearized with *Scal* (lanes 3, 4), *PstI* (lanes 5, 6) or *SmaI* (lanes 7, 8). Proteins were analyzed by 10% polyacrylamide gel electrophoresis. Lanes 9 and 10 are the products of *SmaI*-cut pSG1BF transcript that contains the GAUC duplication in the *BamHI*<sub>4837</sub> site of the 3'TE. Lanes 11 and 12 show translation products of *SmaI*-cut pSP17 transcript in which the 5'-terminal 99 nt of the 188-nt 5' UTR of sgRNA1 were deleted. Mobilities of products of ORFs 3 (22 kDa), 4 (17 kDa) and 3+5 (72 kDa, made by the in-frame read-through of the ORF 3 stop codon) are indicated at right. Other bands indicate cleavage products of the labile 72-kDa protein (Filichkin et al., 1994) and premature termination products within ORF 5 (Brown et al., 1996). Relative moles of translation product (of ORF 3) determined with a Phosphor-imager using ImageQuant software are indicated below each lane. Samples in lanes 9–10 and 11–12 were from different experiments, and the products of the 100% standard (capped *SmaI*-cut pSG1 transcript) for these are not shown. **B:** Products of transcripts from *SmaI*-cut pSG2 (lanes 2, 3) and pSG2BF (lanes 4, 5), following electrophoresis on a 10% polyacrylamide gel. Mobilities of the two smallest BMV RNA translation products (35 and 20 kDa) and the mobility of the ORF 6 product (6.7 kDa) are at left.

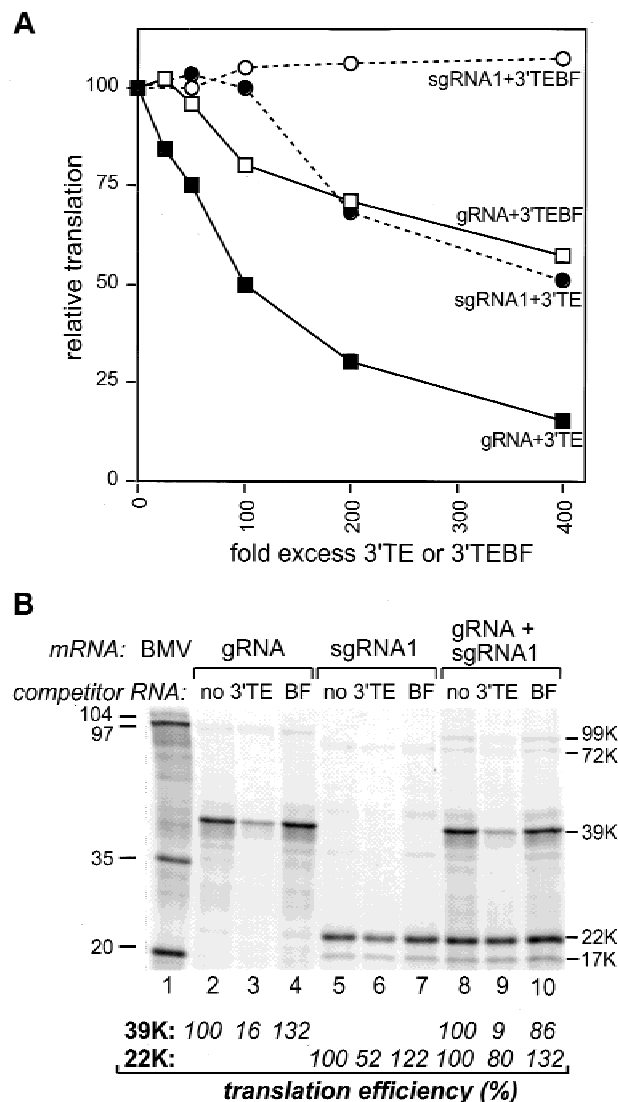
the translation efficiency of wild-type sgRNA2 with that containing the defective 3'TE (with the four-base duplication in the *BamHI*<sub>4837</sub> site). The 6.7-kDa product of ORF 6 was synthesized from uncapped sgRNA2 in

wheat germ extract (Fig. 3B). Most importantly, the four-base duplication in the *BamHI* site abolished translation of uncapped sgRNA2, consistent with our previous observation of reporter gene translation (Wang et al., 1997). Thus, the 3'TE, in the 5' UTR of sgRNA2, facilitates cap-independent translation of ORF 6. As proposed above, the single copy of the 3'TE that is stored in genomic RNA functions on genomic RNA and both sgRNAs 1 and 2.

### 3'TE RNA *trans*-inhibits translation of genomic RNA much more than sgRNA1

Previously, we found that the 109-nt 3'TE, *in trans*, inhibited translation of a reporter gene carrying the 3'TE *in cis* (Wang et al., 1997). Therefore, we tested the ability of the 109-nt 3'TE RNA to inhibit translation of genomic and sgRNA1 *in trans*. A 100-fold molar excess of the 3'TE RNA inhibited translation of gRNA by 50%, whereas four times as much 3'TE RNA was required to inhibit translation of sgRNA1 by 50% (Fig. 4A). The defective 3'TE RNA containing the filled-in *BamHI*<sub>4837</sub> site (3'TEBF RNA) was far less inhibitory of either mRNA (Fig. 4A). A 300-fold excess of 3'TE RNA reduced translation of the 39-kDa product of ORF 1 from gRNA by sixfold (Fig. 4B, lanes 2–3), whereas translation of coat protein from sgRNA1 was only halved (Fig. 4B, lanes 5–6). Most strikingly, when equal amounts of genomic and sgRNA1 were present in the same reaction, presence of excess 3'TE RNA dropped gRNA translation by 11-fold, whereas translation of sgRNA1 was reduced by only 20% (Fig. 4B, lanes 8–9). In all cases, the defective 3'TE had little effect on the translation from genomic RNA or sgRNA1 *in trans*. (The apparent inhibition of gRNA by 3'TEBF RNA in Fig. 4A and apparent stimulation in Fig. 4B, lane 4, reflects experimental variation ( $\pm 32\%$ ). Inhibition greater than twofold is considered significant.) Thus, the *trans*-inhibition requires a functional 3'TE sequence and it specifically inhibits gRNA much more than sgRNA1.

sgRNA2 accumulates to a 20–40-fold molar excess over genomic RNA in infected cells (Kelly et al., 1994; Mohan et al., 1995; Koev et al., 1998). The ratio of sgRNA2 to *translatable* gRNA is even greater than that seen on Northern blots, because much of the genomic RNA is encapsidated (Mohan et al., 1995) and thus sequestered from translation. Because the 3'TE comprises the complete 5' UTR of sgRNA2, it is possible that sgRNA2 inhibits translation of genomic and sgRNA1 *in trans*. Because of the preferential inhibition of gRNA versus sgRNA1, we propose that as sgRNA2 accumulates, translation of gRNA is reduced, favoring translation of sgRNA1 late in infection. To test this hypothesis, the effect of sgRNA2 on translation of gRNA and sgRNA1 was evaluated as in the previous experiment. As predicted, sgRNA2 inhibited translation of the genomic RNA more effectively than it inhibited translation

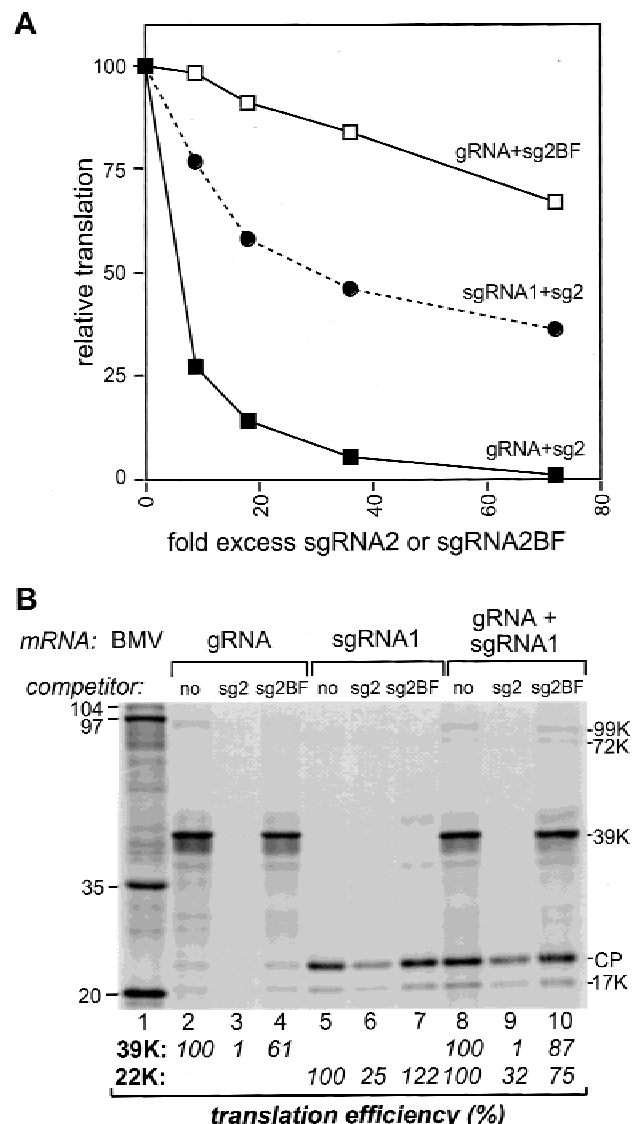


**FIGURE 4.** Differential effects of 109-nt wild-type and mutant 3' TE RNAs *in trans* on translation of gRNA and sgRNA1. **A:** Indicated amounts of 109-nt 3' TE transcript from *Sma*I-cut p3TE or 113-nt transcript from p3TEBF (Wang et al., 1997) were mixed with 0.1 pmol PAV6 or SG1 transcripts and translated in wheat germ extract. Following electrophoresis, products were quantitated by phosphorimager to determine relative translation. **B:** Translation of 0.1 pmol gRNA (lanes 2–4, 8–10), and/or 0.1 pmol sgRNA1 (lanes 5–10) in the presence of no 3' TE transcript (lanes 2, 5, 8), 30 pmol 3' TE RNA (lanes 3, 6, 9), or 30 pmol 3' TEBF RNA (lanes 4, 7, 10).

of sgRNA1 (Fig. 5A). As with the inhibition by the 3' TE alone, the *Bam*HI<sub>4837</sub> fill-in mutation in sgRNA2 drastically reduced its ability to inhibit translation of gRNA *in trans* (Fig. 5A). sgRNA2 was more than ten times as effective as the 109-nt 3' TE in inhibiting translation *in trans* (compare Fig. 4A with Fig. 5A). Less than nine-fold excess sgRNA2 inhibited genomic RNA translation by 50%, but about 30-fold excess was required for similar inhibition of sgRNA1 translation (Fig. 5A).

To mimic the scenario in the virus-infected cell gRNA, sgRNA1 and sgRNA2 were mixed in various combina-

tions and translated in wheat germ extract. Wild-type sgRNA2 reduced translation of genomic RNA by 100-fold, whereas translation of sgRNA1 was reduced by three- to fourfold (Fig. 5B). sgRNA2 containing the *Bam*HI fill-in defect had little effect (Fig. 5B, lanes 4, 7, and 10). Thus, the mechanism by which sgRNA2 inhibits translation is 3' TE-mediated. Most interestingly, it is quite feasible that sgRNA2, at physiological ratios, specifically inhibits translation of gRNA in preference to sgRNA1 in the infected cell.

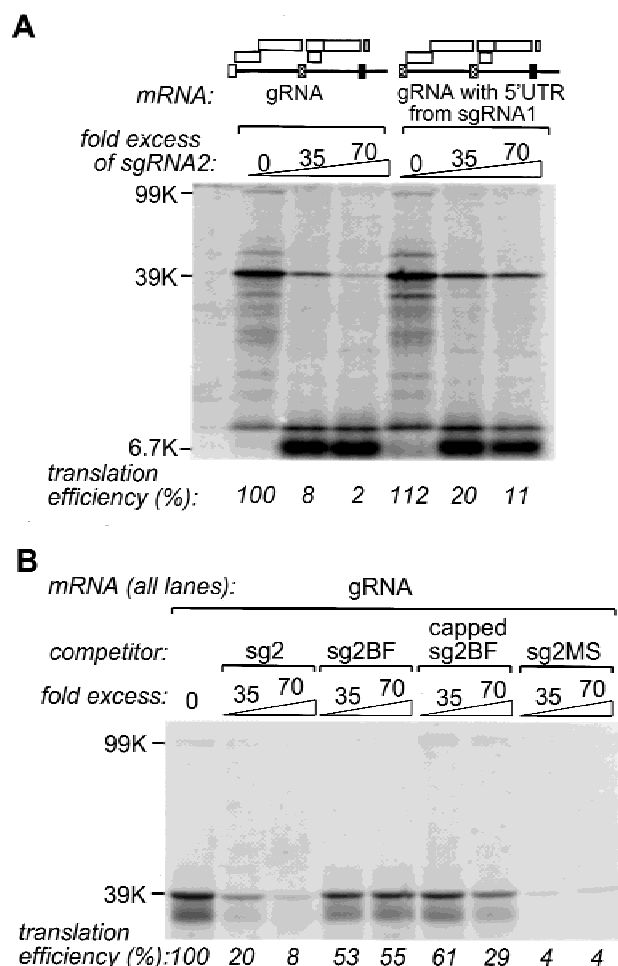


**FIGURE 5.** Differential effects of wild-type and mutant sgRNA2 *in trans* on translation of gRNA and sgRNA1. **A:** Translation of gRNA and sgRNA1 as in Figure 4A but in the presence of increasing molar ratios of transcripts from *Sma*I-cut pSG2 or pSG2BF. Note the lower molar ratios of sgRNA2 used here compared to 3' TE RNAs in Figure 4A. **B:** Translation of 0.1 pmol gRNA (lanes 2–4, 8–10) and/or 0.1 pmol sgRNA1 (lanes 5–10) in the presence of no sgRNA2 transcript (lanes 2, 5, 8), 7 pmol sgRNA2 RNA (lanes 3, 6, 9), or 7 pmol sgRNA2BF RNA (lanes 4, 7, 10).

## Roles of specific RNA sequences in differential *trans*-inhibition

The differential *trans*-inhibition of translation could be due to the longer distance and intervening ORFs between the *cis*-acting 3'TE and the ORF1 start codon on gRNA compared to the shorter distance between the 3'TE and the start codons of ORFs 3 and 4 on sgRNA1. Another possibility is that the different 5' UTR sequences on gRNA and sgRNA1 could have different efficiencies of interaction with the 3'TE. Thirdly, both of the above possibilities could contribute to the differential inhibition by sgRNA2. To test the role of the 5' UTR, the genomic 5' UTR was replaced with the 5' UTR of sgRNA1. This modified gRNA translated with efficiency similar to wild-type gRNA, but it was inhibited less by sgRNA2 *in trans* than was wild-type gRNA (Fig. 6A). Thus, the differential susceptibility to *trans*-inhibition by sgRNA2 is at least partially due to the sequences of the 5' UTRs. The closer proximity of the 3'TE to the start codon may also allow more efficient cap-independent translation, as we observed a stronger stimulatory effect in reporter constructs in which the 3'TE was immediately 3' of the stop codon compared to gRNA (Wang & Miller, 1995). Thus, we conclude that both the nature of the 5' UTR sequence and the proximity of the 3'TE to the start codon contribute to the preferential *trans*-inhibition of translation of gRNA versus sgRNA1 by sgRNA2.

Another question is why full-length sgRNA2 inhibits so much more effectively than the 109-nt 3'TE RNA. Either the product of ORF 6 or simply the act of translation, in which sgRNA2 competes with gRNA and sgRNA1 for the translational apparatus, may account for the greater inhibition by sgRNA2. To test these possibilities, we measured the inhibition of gRNA translation by capped sgRNA2BF. This RNA is an efficient message (Fig. 3B, lane 4) but it inhibited gRNA translation only moderately (Fig. 6B, capped sg2BF). This inhibition was the same as, or only slightly more than, the inhibition by uncapped sgRNA2BF (Fig. 6B, sg2BF). We then examined the inhibitory activity of a mutant sgRNA2 lacking a start codon but containing a wild-type 3'TE. No other AUGs exist in any frame in the ORF 6 sequence, so no ORF 6 product could be made. This RNA inhibited translation of genomic RNA at least as effectively as wild-type sgRNA2, if not more so (Fig. 6B, lanes sg2 and sg2MS). We conclude that neither translation of sgRNA2 per se nor the ORF 6 product inhibit translation of gRNA. Instead, a wild-type 3'TE sequence, combined with the additional sgRNA2 sequence, is the component that confers shutoff of genomic RNA translation. Thus, the inhibition is independent of the translatability of sgRNA2, and independent of the presence of a 5' cap on sgRNA2.



**FIGURE 6.** Inhibition of genomic RNA translation by sgRNA2. **A:** Effects of sgRNA2 on translation of wild-type genomic RNA with its natural 5' UTR or genomic RNA containing the 5' UTR from sgRNA1 in place of its 5' UTR. Maps of transcripts are indicated above lanes. Open box: 5' UTR of genomic RNA; stippled box: 5' UTR of sgRNA1; black box: 3'TE. Wheat germ translation products were analyzed by 10% polyacrylamide gel electrophoresis. Left lane: no RNA. Other lanes have 0.1 pmol of the indicated form of gRNA with the indicated molar excess of sgRNA2 (from *Sma*I-cut pSG2). The 5' UTR of gRNA (bases 1–148) was replaced with that from sgRNA1 (bases 2670–2860) in transcript from pSG1PAV6 (right three lanes). Mobilities of products of ORF 6 (6.7K), ORF 1 (39K) and ORFs 1+2 (99K) are at left. **B:** Wheat germ translation products of 0.1 pmol uncapped PAV6 RNA in the presence of indicated molar excess of uncapped sgRNA2 (sg2), capped and uncapped, *Bam*HI-filled-in mutant sgRNA2 (sg2BF), and uncapped sgRNA2 with the mutant (AUG → AUC) ORF 6 start codon (sg2MS). Product of sgRNA2 ran off the bottom of the gel.

## DISCUSSION

### The sgRNA2 sequence is sufficient, *in cis*, for cap-independent translation in vivo and inhibits efficiently *in trans*

We confirmed and refined the previous observation (Wang et al., 1997) that more sequence from the 3' end of the BYDV genome is necessary for cap-independent translation in vivo than in vitro. The fact that the 3'TE



functions efficiently in the 3' UTRs of both luciferase (Fig. 2) and GUS (Wang et al., 1997) reporter genes verifies that the cap-independent translation activity is independent of the coding region. This is significant because, in some cases, different reporter genes can give different results (Gallie et al., 1991).

The role of the additional sequence needed *in vivo* but not *in vitro* is unknown. One possibility is that the additional viral sequence mimics a poly(A) tail. BYDV RNA is not polyadenylated. Wheat germ translation extracts are virtually poly(A) tail-independent, whereas the poly(A) tail plays a crucial role in translation initiation *in vivo* (Gallie, 1991; Hentze, 1997; Sachs et al., 1997; Preiss & Hentze, 1998). A pseudoknot-rich domain has been identified in the 3'UTR of TMV that functionally substitutes for a poly(A) tail (Gallie & Walbot, 1990). Together with a 5' cap, it synergistically stimulates translation of mRNAs (Gallie, 1991). Such a function (with a different structure) may exist between bases 5010 and 5320 in the 3' UTR of BYDV, because deletion of this region substantially and equally reduced translation of capped and uncapped mRNAs in protoplasts (Fig. 2). However, additional cap-independent translation functions must exist outside of the 109-nt 3'TE region, because *in vivo* translation of a construct containing only the 109-nt 3'TE plus a 30-nt poly(A) tail in its 3'UTR was stimulated 10-fold by addition of a 5' cap (Wang et al., 1997). The additional sequence may be limited to the five bases at positions 4809–4513, or sequence between nt 4922 and 5010 may also contribute to cap-independent translation *in vivo* (Fig. 2).

A different structure in the 3' UTR of alfalfa mosaic virus RNA 4 enhances the ability of mRNAs to compete in cap-dependent translation (Hann et al., 1997). The competitive environment in a cell is quite different from that in wheat germ extract and may explain the need for additional BYDV 3'UTR sequence for cap-independent translation.

The more efficient *trans*-inhibition of translation by full-length sgRNA2 than the 109-nt 3'TE is not due to the active translation of sgRNA2, because mutation of the ORF 6 start codon had no effect on *trans*-inhibition (Fig. 6B). Furthermore, translatable (capped) sgRNA2 with a defective 3'TE (Fig. 3B) did not inhibit *in trans* (Fig. 6B). Thus, like the 109-nt 3'TE alone, sgRNA2 inhibits via the 3'TE-mediated mechanism. We speculate the sgRNA2 inhibits more efficiently because it may have a higher binding affinity for protein factors that mediate cap-independent translation. This could also explain the need for the sgRNA2 sequence *in cis* for cap-independent translation *in vivo*.

### sgRNA2 may facilitate a switch from early to late gene expression

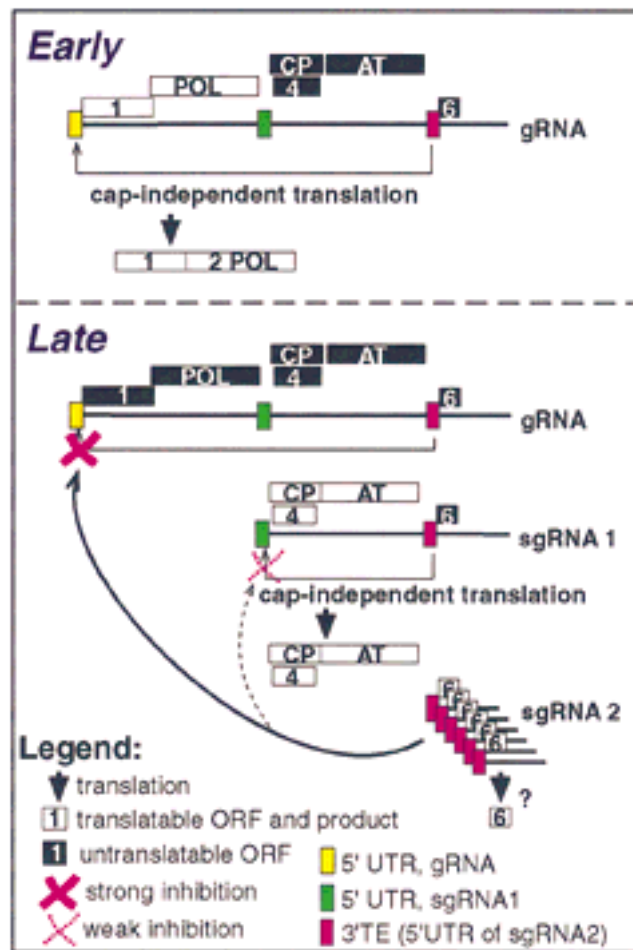
Gene expression of many viruses is divided into temporal stages with nonstructural replication proteins ex-

pressed early and structural proteins expressed late. Synthesis of BYDV subgenomic RNAs requires replication, so the structural genes they encode are not translated until after RNA replication has commenced. Thus, RNA-templated transcription (subgenomic RNA synthesis) alone can account for turning on late gene expression. However, the data presented here suggest an additional level of control mediated by viral RNA *in trans* that may act to shut off expression of early genes.

We propose a model of *trans*-regulation of translation by the 3'TE in which accumulation of sgRNA2 at high levels preferentially inhibits translation of genomic RNA over sgRNA1. Early in infection, genomic RNA from the invading virion is the only message (*Early*, Fig. 7). This allows cap-independent translation of ORFs 1 and 1+2 (replicase) facilitated by the 3'TE *in cis*. The replicase then replicates gRNA and transcribes sgRNAs. As large amounts of sgRNA2 accumulate (*Late*, Fig. 7), it strongly inhibits translation of gRNA, shutting off translation of replication genes (ORFs 1 and 2), while only weakly inhibiting translation of sgRNA1, permitting translation of structural and movement protein genes (ORFs 3, 4, and 5).

This model is supported by the following observations. (1) The 3'TE is required *in cis* for translation (Allen et al., 1999) of the only two genes (ORFs 1 and 2) required for RNA replication (Mohan et al., 1995). (2) Thus, intact 3'TE is required for replication *in vivo* (Allen et al., 1999). (3) Only ORFs 1 and 2 are translated from gRNA (Di et al., 1993; Mohan et al., 1995; Allen et al., 1999). (4) The 5' end of the *in vivo*-defined 3'TE sequence that gives cap-independent translation *in cis* coincides precisely with the 5' end of sgRNA2 (Fig. 2). (5) sgRNA2 inhibits translation of gRNA *in trans* far more efficiently than it inhibits translation of sgRNA1 (Fig. 5A). (6) When gRNA and sgRNA1 are competing with each other in the presence of sgRNA2 at ratios similar to those in infected cells, only the products of sgRNA1 are translated significantly, and gRNA is virtually shut off (Fig. 5B). sgRNA2 accumulates to at least 20- to 40-fold molar excess to gRNA (Kelly et al., 1994; Mohan et al., 1995; Koev et al., 1998) and probably to a higher ratio when compared to translatable (non-encapsidated) gRNA.

The proposed mechanism in Figure 7 can be compared to other known viral translational control mechanisms. Subgenomic mRNA synthesis from genomic RNA1 of red clover necrotic mosaic virus is controlled by direct base-pairing of genomic RNA2 to RNA1 (Sit et al., 1998), but this is an example of (RNA-templated) transcription rather than translation. RNA phages Q $\beta$  and MS2 use long-distance base pairing *in cis* to negatively regulate translation of the A protein and replicase. The replicase and coat proteins act *in trans* to shut off translation of each other's genes. This facilitates switches from translation to replication and from replication to encapsidation (Weber et al., 1972; van



**FIGURE 7.** Translational switch model for *trans*-regulation of BYDV gene expression by sgRNA2. Open boxes indicate translatable ORFs, and their translation products (below large arrows). Black boxes indicate ORFs that are not translated. *Early*: polymerase is translated from gRNA (the only viral RNA at this stage) via the 3'TE (red box) *in cis*. As abundant sgRNA2 accumulates (*Late*), it specifically inhibits gRNA (bold red X) in preference to sgRNA1 (dashed red X), via the 3'TE *in trans*. This allows almost exclusive translation of late genes from sgRNA1. The different 5' UTRs of gRNA (gold box) and sgRNA1 (green box) contribute to the differential inhibition. The role of ORF 6 encoded by sgRNA2 is unknown (?), but it is not necessary for *trans*-inhibition. See text for detailed discussion of the model.

Duin, 1988). In adenovirus-infected cells, late (structural) gene expression coincides with dephosphorylation of eIF4E that inhibits cellular cap-dependent translation, and favors translation of viral mRNAs that have reduced cap-dependence (Schneider, 1995; Kleijn et al., 1996; Gingras & Sonenberg, 1997; Sonenberg & Gingras, 1998). All of these mechanisms and known mechanisms of translational control of host genes involve regulation by protein binding, modulation of translation factors, or antisense RNA. In contrast, sgRNA2 is a sense RNA that, we propose, differentially controls translation. It is quite possible that the sgRNA2 inhibits by competing for a protein such as a translation factor needed for 3'TE-mediated translation (Wang et al.,

1997). Regardless of the specific components involved, this appears to be a novel type of gene expression control in which a truncated form of an mRNA converts a *cis*-stimulatory sequence into a *trans* inhibitor, as a natural means of differential translational control.

The 5'UTRs of gRNA and sgRNA1 appear to be at least in part responsible for the differential inhibition by sgRNA2 (Fig. 6A). This is likely due to differences in their ability to communicate with the 3'TE, and not due to an inherent difference in ability to recruit ribosomes or initiation factors directly, because the natural 5'UTR is dispensable when the 3'TE is located in the 5'UTR (Fig. 3B and Wang et al., 1997). Thus, the sgRNA1 5'UTR would be predicted to have a higher affinity for the 3'TE, probably mediated by protein factors, than would the 5'UTR of gRNA.

### Advantages of cap-independent translation

In addition to facilitating the proposed regulatory model, cap-independent translation has other innate advantages. The initiation factor involved in cap recognition, eIF4E, is considered to be the rate-limiting factor for translation initiation (Sonenberg, 1996). Thus, viral RNAs that have a reduced requirement or no requirement for eIF4E could be translated more efficiently. This is the case for cap-independently translated viruses such as picornaviruses (Pestova et al., 1996) and pestiviruses (Pestova et al., 1998), and the late viral genes of adenovirus (Schneider, 1995). This gives the viral mRNAs a competitive advantage, especially under stressed conditions in which translation of capped cellular mRNAs is often shut down by modulation of eIF4E (Sonenberg, 1996), or by the virus itself (Pestova et al., 1996). Like the above viruses, translation initiation mediated by the 3'TE has reduced dependence on eIF4F (which consists of eIF4E bound to eIF4G; Browning, 1996) (Wang et al., 1997), so BYDV RNA should have a competitive advantage as well. A cap-independent translation sequence in the 3' UTR of STNV RNA also lowers the requirement for eIF4F (Timmer et al., 1993). The 5' UTRs of tobacco mosaic virus (Sleat et al., 1987) and potato virus X (Zelenina et al., 1992), and the 3' UTR of alfalfa mosaic virus (Hann et al., 1997) also give competitive translational advantages to their mRNAs, but in a cap-dependent (and thus eIF4F-dependent) fashion.

Another obvious advantage of cap-independent translation is the lack of a requirement for a methyltransferase encoded by the virus. The host methyltransferase activity required for N7-methylation of the guanosine in the cap is located in the nucleus. Many cytoplasmic RNA viruses such as tricoronaviridae and alphaviruses code for their own methyltransferase enzymes to cap their RNA (Koonin & Dolja, 1993). BYDV does not appear to code for such an enzyme (Koonin & Dolja, 1993), which allows for a simpler, smaller genome, giving it a replicative advantage.

This role of a sense RNA as a regulatory switch (Fig. 7) would be a new example of the apparently infinite variety of means by which viruses regulate gene expression. Obviously, we must now test this mechanism in a natural infection, and identify the protein components involved in the 3' TE mechanism. Initial *in vivo* evidence supports the model: a point mutation that blocked accumulation of sgRNA2 reduced, but did not eliminate, virus replication in protoplasts (Mohan et al., 1995). We must also examine the possibility that sgRNA2 may cause disease by *trans*-inhibiting host translation. The specific and differential effects on translation shown here reveal the importance of considering RNAs as potential specific *trans*-regulators of translation. This principle could potentially apply to control of other viral and possibly host genes.

## MATERIALS AND METHODS

### Plasmid construction

All constructs were verified by automated sequencing at the ISU Nucleic Acids Facility on an ABI 377 sequencer. Construction of plasmids pTE and pTEBF was described by Wang et al. (1997), pPAV6 by Di et al. (1993), and pSP17 by Dinesh-Kumar et al. (1992). pPAV6BF was constructed in three steps. First, a *KpnI*–*SmaI* fragment from pPAV6 was cloned into *KpnI*–*SmaI* digested pGEM3Zf(+) (Promega, Madison, WI), giving rise to p3ZKS. p3ZKS was cut with *Bam*HI and filled in with Klenow fragment and religated, giving rise to p3ZKSBF. Finally the *KpnI*–*SmaI* fragment from p3ZKSBF was cloned back into pPAV6 digested with the same restriction enzymes. Plasmid pSG1, for transcription of sgRNA1, was constructed by PCR amplification of pPAV6 using the primer, subgen1-up: ATAAGCGGCCGCGTAATACGACTCACTATAGTGAAGGTGACGACTCCACATC, which corresponds to the 5' end of sgRNA1 (bases 2670–2691), and the downstream primer, SK020601: GGGCCCGGGTTGCCGAACTGCTCTTTTCG, which anneals to the 3' end of genomic RNA (nt 5677–5656) (restriction sites are underlined, and the T7 promoter is italicized). The PCR product was digested with *NotI* and *SmaI* and cloned into *NotI*–*SmaI* digested pSL1180 (Pharmacia). The same strategy was used to construct the mutant sgRNA1 plasmid, pSG1BF, using pPAV6BF as the PCR template.

Plasmid pSG2 for T7 transcription of sgRNA2 was constructed by amplifying pPAV6 with primer, subgen2-up: TATTGCGGCCGCGTAATACGACTCACTATAGAGTGAAGACAACACTAGCAC, which corresponds to BYDV genome bases 4809–4831, and the downstream primer, SK020601. (An extra guanosine nucleotide was placed 5' of the start of subgenomic RNA2 to facilitate *in vitro* transcription.) The PCR product was digested with *NotI* and *SmaI* and cloned into pSL1180 cut with the same restriction enzymes, giving rise to pSG2. The same cloning strategy was used to obtain pSG2BF and pSG2MS, except the templates for PCR were pPAV6BF and pPAV30 (Mohan et al., 1995), respectively. pSG2MS differs from pSG2 by a single G-to-C substitution that changes the ORF 6 start codon to AUC.

To replace the genomic 5' UTR with that of sgRNA1 (plasmid pSG1PAV6), the subgenomic RNA1 5' UTR was PCR

amplified from pPAV6 using the primer 5'UTRswap (GCG TTCGAAGAACAATTCACCACCTCTCTAGTGG), which contains a *Csp45I* site (underlined) followed by sequence complementary to bases 2860–2840) and the primer subgen-up. Both the PCR product and pPAV6 were digested with *NotI* and *Csp45I*. *Csp45I* cuts just downstream of the ORF 1 start codon in pPAV6. These DNAs were gel-purified and ligated together, resulting in plasmid pSG1PAV6.

### pLUC plasmids

pPAV6 was modified at three bases to introduce a unique *Bss*HII site just 5' of the ORF 1 start codon. A pair of PCR primers (5' primer contains a *Bss*HII site, and 3' primer contains an *Acc*65I site) was used to amplify the firefly luciferase (LUC) coding region from pGEM-luc (Promega). After digestion with *Bss*HII and *Acc*65I, this fragment was cloned into *Bss*HII–*Acc*65I–cut pPAV6, replacing bases 138–4153 (ORFs 1–4 and part of 5) of the BYDV genome with the LUC gene. This resulted in plasmid pLUC1524, which has the 5' UTR of BYDV, LUC gene, and the 3'-terminal 1,524 nt of BYDV sequence. (This series of plasmids is named for the LUC gene followed by the number of bases from the 3' end of the BYDV genome that are in its 3' UTR.) The set of constructs containing nested 5' terminal deletions of the series of the 3'-terminal sequence was made from this construct. Specifically, the deletion series was amplified by PCR (5' primers contained an *Acc*65I site followed by 17 bases of PAV sequence at the desired deletion site, 3' primer was SK020601). The series of PCR products was cut with *Acc*65I and *SmaI* and cloned into pLUC1524 cut with the same enzymes.

### RNA preparation

The uncapped and capped RNAs were synthesized by transcription with T7 polymerase using the MegaScript or mMessage mMachine kits (Ambion, Austin, TX) as described previously. All transcripts are named for their parent plasmid minus the lower case p prefix. The RNA concentration was determined with a spectrophotometer. Integrity was verified by 1% agarose gel electrophoresis.

### In vitro translation

Nonsaturating amounts of RNAs were translated in wheat germ extract (Promega) according to manufacturer's instructions in a total volume of 25  $\mu$ L (Wang & Miller, 1995). In RNA competition experiments, the mRNA was mixed with the competitor RNA prior to adding into the translation reaction. Five microliters of translation product were separated on 10% SDS-PAGE gel (Wang & Miller, 1995). The relative translation efficiency was determined by quantification of the major translation products (39K or 22K) with the ImageQuant™ program.

### Luciferase assays

Three picomoles transcript were electroporated into 10<sup>6</sup> oat protoplasts as for transfection as in Wang et al. (1997). After 20 h, protoplasts were collected and lysed in 100  $\mu$ L Passive



Lysis Buffer (Promega) by shaking 15 min at room temperature. Fifty microliters luciferase substrate Luciferase Assay Reagent II (Promega) were mixed with 10  $\mu$ L protoplast lysate supernatant and measured on a Turner Designs TD-20/20 luminometer. Protein concentration of each sample was measured by the Bradford method (Bio Rad) to normalize luciferase activity for each sample.

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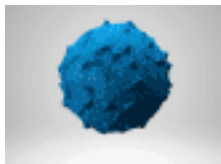
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